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Stretch activation of Jun N-terminal kinase/stress-activated protein kinase in mesangial cells

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Background. Mesangial cells (MCs) grown on extracellular matrix (ECM)-coated plates and exposed to cyclic stretch/relaxation proliferate and produce ECM protein, suggesting that this may be a useful in vitro model for MC behavior in response to increased physical forces. The induction of c-*fos* in response to MC stretch has been shown. Stimuli that lead to c-*fos* induction pass through mitogen-activated protein (MAP) kinase pathways. We have seen early activation of jun N-terminal kinase/stress-activated protein kinase (SAPK/JNK) in MCs exposed to cyclic stretch. Accordingly, we studied SAPK/JNK activation in stretched MCs and the downstream consequences of this signaling.

Methods. MCs (passages 5 to 10) cultured on type 1 collagen-coated, flexible-bottom plates were exposed to 2 to 60 minutes of cyclic strain (60 cycles per minute) by generation of vacuums of −10 to −27 kPa, inducing approximately 16 to 28% maximum elongation in the diameter of the surfaces. Control MCs were grown on coated rigid bottom plates. Protein levels (by Western blot) and activity assays for SAPK/JNK were performed under these conditions. We observed marked activation at −18 kPa and above and at two minutes, and then we studied activation mechanisms under these conditions. Nuclear protein binding to activator protein-1 (AP-1) consensus sequences was also examined. The role of calcium was studied with EGTA and BAPTA-AM to chelate extra- and intracellular calcium, respectively. Protein kinase C (PKC) was down-regulated by incubation with phorbol ester (PMA) for 24 hours prior to stretch. In unstretched MCs, A23187 was used as a calcium ionophore, and PKC was up-regulated with PMA application for 30 minutes to determine the effects on SAPK/JNK. Nuclear protein binding to AP-1 was also determined under these conditions. The effects of stretch, acute PMA, and A23187 on fibronectin mRNA levels were studied using reverse transcriptase-polymerase chain reaction (RT-PCR).

Results. Cyclic strain/relaxation led to increased SAPK/JNK activity only at two minutes and −18 kPa and above. The activation of SAPK/JNK was dependent on intracellular calcium, with BAPTA-AM almost completely abrogating the response to stretch. EGTA was without effect. Down-regulation of PKC also led to a diminution of activity. In static cells, the calcium ionophore A23187 increased SAPK/JNK activity, and this was potentiated by acute PMA. Stretch, acute PMA, and A23187 all increased nuclear protein binding to AP-1 consensus sequences. mRNA levels for fibronectin were increased by stretch in MCs and by PMA and A23187 in static MCs. No change was observed in the amount of SAPK/JNK protein present in stretched MCs by Western blot.

Conclusions. Stretch leads to early activation of SAPK/JNK in MCs. This is dependent on intracellular calcium and PKC and can be replicated by activation of these stimuli in static MCs. A downstream induction of nuclear protein binding to AP-1 consensus sequences was seen in a pattern that was completely concordant with the SAPK/JNK induction.

Key words: mechanical strain, calcium, extracellular matrix protein, protein kinase C, cyclic stretch, signaling, nuclear protein binding.

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Glomerular mesangial cells (MCs) experience pulsatile stretch/relaxation [1] as a result of exposure to a microcirculation with vascular pressures greater than those of any other capillary bed [2]. Despite this mechanical force of about 45 mm Hg, little resident glomerular cell proliferation or sclerosis is demonstrable in intact animals. Conversely, an increase of about 10 mm Hg over this level results in glomerular cell proliferation and the accumulation of extracellular matrix (ECM) protein in several animal models of glomerular sclerosis [3–5]. Moreover, maneuvers that decrease intraglomerular pressure attenuate sclerotic injury in these models, further implicating mechanical forces [5–7].

The effects of mechanical forces on MCs in vitro have been studied by culturing cells on plates with deformable bottoms and applying vacuum to the well to generate alternating cycles of stretch and relaxation. Initial experiments using this methodology showed increases in cellular calcium entry and total protein kinase C (PKC) activity within five minutes of the application of stretch to MCs [1], followed by induction of mRNA for the proto-oncogene and activator protein-1 (AP-1) transcription factor component c-*fos* at 30 minutes [1]. This effect was blocked by PKC inhibition [1]. Subsequently, increases in both MC proliferation [2] and collagenous and noncollagenous ECM protein synthesis were observed by 48 hours, the sine qua non of sclerotic injury [8, 9]. Trans-

forming growth factor-β1 (TGF-β1) has been implicated in the accumulation of ECM in glomeruli under these conditions [10–12], which could be blocked with inhibitors of tyrosine kinase [13]. Since the TGF-β1 promoter region contains two AP-1 consensus sequences in a positive regulatory region [14, 15] and c-*fos* mediates TGF-β1 autoinduction [16], it is likely that TGF-β1 gene expression in response to stretch is at least partly dependent on AP-1 transcription factor activity.

We and others have studied the link between early events such as PKC activation and induction of transcription of c-*fos* and TGF-β1 in stressed MCs [13, 17, 18]. Early increases in p44/42 (ERK) mitogen-activated protein kinase (MAPK) signaling were observed in response to constant pressure, an effect that could be abrogated by tyrosine kinase inhibition [17]. We demonstrated increases in p44/42 and p38 HOG signaling in response to cyclic stretch at 30 minutes [18]. We did not observe any jun N-terminal kinase/stress-activated protein kinase (SAPK/JNK) activity at these time points. However, several recent publications have demonstrated SAPK/JNK activity within minutes of application of physical force to vascular smooth muscle cells (VSMCs) [19] or cardiac myocytes [20]. The very early activation of SAPK/JNK has led to an examination of the induction of this phenomenon. In response to angiotensin II (Ang II) applied to cardiac myocytes, PKC and intracellular calcium were found to play necessary roles in induction of SAPK/JNK activity [21]. Stretch-induced SAPK/JNK activation was not PKC dependent in the same cells, however [20]. Mechanisms of SAPK/JNK activation in response to cyclic mechanical stress have not been studied in MCs; however, we did observe early (<5 minutes) activity in the SAPK signaling pathway in preliminary work (unpublished observations).

Accordingly, we sought to characterize the SAPK/JNK activation we have seen in response to cyclic MC stretch and to determine how the stretch signal might be transduced. We also sought to relate these observations to downstream events by studying nuclear protein binding to AP-1 consensus sequences in stretched MCs.

METHODS

Cell culture

Sprague-Dawley rat MCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS; GIBCO BRL, Grand Island, NY, USA), streptomycin (100 μg/mL), penicillin (100 U/mL), and 2 mmol/L glutamine at 37°C in 95% air/5% CO_2. Experiments were carried out in cells between passages 5 and 10.

Application of stretch/relaxation

Mesangial cells (2×10^6/well) were plated on to six-well plates with either a rigid or flexible-bottom coated with bovine type I collagen (Flexcell International Corp., McKeesport, PA, USA). Cells were grown to confluence for 72 hours and then rendered quiescent by incubation for 24 hours in DMEM with 0.5% FCS. To characterize the time of maximum response, cells on the flexible-bottom plates were initially exposed to cycles of stretch/relaxation for periods of 2, 5, 10, 30, and 60 minutes generated by a cyclic vacuum generated by a computer-driven system (Flexercell Strain Unit 2000; Flexcell Co.). Plates were exposed to continuous cycles of stretch/relaxation, with each cycle being 0.5 seconds of stretch and 0.5 seconds of relaxation, for a total of 60 cycles per minute. Initially, vacuum pressures used were −10 to −27 kPa, inducing a 16 to 27% maximum elongation in the diameter of the surface. Subsequent experiments were performed at the time of maximal response, two minutes and at −18 kPa.

To characterize the early events involved in SAPK/JNK signaling, calcium and PKC inhibition and stimulation were studied. Extracellular and intracellular calcium were chelated by the addition of EGTA 1 mmol/L or BAPTA-AM 10 μmol/L, respectively, for 30 minutes prior to the application of stretch. PKC was down-regulated by incubation with 100 nmol/L phorbol ester (PMA) for 24 hours prior to stretching. Stimulation experiments in static MCs used A23187 1 μg/mL as a calcium ionophore and PMA 1 μmol/L for 30 minutes to activate PKC. In all experiments involving SAPK/JNK activity, sorbitol 400 mmol/L for 30 minutes was used as an osmotic stimulus positive control.

Protein isolation

Cellular levels of SAPK/JNK protein were determined in stretched and unstretched control cells at the indicated times and vacuum levels after the application of stretch and then subsequently with inhibitors and agonists added prior to two minutes of stretch at −18 kPa. Briefly, at the end of each stretch protocol, media were removed, and the cells were washed once with ice-cold phosphate-buffered saline (PBS). PBS was then removed, and cells were harvested under nondenaturing conditions on ice by incubation for five minutes with 0.5 mL 1 × ice-cold cell lysis buffer [20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L egtazic acid (EGTA), 1% triton, 2.5 mmol/L Na pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na orthovanadate, 1 μg/mL leupeptin] and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Cells were then scraped into microcentrifuge tubes on ice and sonicated four times for five seconds each. After microcentrifugation at 14,000 r.p.m. for 10 minutes at 4°C, the supernatant was transferred to a fresh microcentrifuge tube. Protein concentration was measured with the Bio-Rad assay kit.

Western blotting for JNK/SAPK

A 40 μg sample was then separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electroblotting to a nitrocellulose membrane (Protran; Schleicher and Schuell, Keene, NH, USA), membranes were incubated for three hours at room temperature with 25 mL of blocking buffer (1 × TBS, 0.1% Tween-20 with 5% wt/vol nonfat dry milk) and then overnight at 4°C with SAPK/JNK polyclonal antibody (1:1000; New England Biolabs, Beverly, MA, USA) in 10 mL of antibody dilution buffer [1 × TBS, 0.05% Tween-20 with 5% bovine serum albumin (BSA)] with gentle rocking overnight at 4°C. Membranes were then washed three times with TTBS and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:2000) in 10 mL of blocking buffer for 45 minutes at room temperature. After three further Tris-buffered saline washes, the membrane was incubated with LumiGlo reagent (KPL Inc., Gaithersburg, MD, USA) and then exposed to x-ray film (X-OMAT; Kodak, Rochester, NY, USA).

"Pull-down" SAP kinase assay

After protein isolation as mentioned previously in this article, 2 μg of c-Jun fusion protein beads (New England Biolabs) were added to 250 μg of cell lysate protein and incubated overnight at 4°C. Lysate was then centrifuged for 30 seconds to recover the beads and was washed twice with 0.5 mL of 1 × lysis buffer. For the kinase assay, pellets were washed twice with 0.5 mL kinase buffer [25 mmol/L Tris, 5 mmol/L β-glycerophosphate, 2 mmol/L dithiothreitol (DTT), 0.1 mmol/L Na orthovanadate, 10 mmol/L $MgCl_2$]. After incubation for 30 minutes at 30°C, the reaction was terminated with 25 μmol/L 3 × SDS sample buffer (187.5 mmol/L Tris-HCl, pH 6.8, 6% wt/vol SDS, 30% glycerol, 150 mmol/L DTT, 0.3% wt/vol bromphenol blue), boiled for five minutes, vortexed, and then microcentrifuged for two minutes. Twenty microliters of sample were run on a 12% SDS-PAGE gel. After blotting to nitrocellulose, membranes were incubated for three hours at room temperature with 25 mL of blocking buffer (1 × TBS, 0.1% Tween-20 with 5% wt/vol nonfat dry milk) and then overnight at 4°C with a phospho-specific c-Jun (ser63) antibody at 1:1000 dilution in 10 mL of antibody dilution buffer (1 × TBS, 0.05% Tween-20 with 5% BSA). Gels were washed three times with TTBS and then incubated with HRP-conjugated antirabbit secondary antibody (1:2000) for one hour at room temperature. After three Tris-buffered saline washes, the membrane was incubated with Lumi-Glo reagent (KPL Inc.) and then exposed to x-ray film (X-OMAT; Kodak).

Nuclear protein binding to AP-1 consensus sequences

These experiments were performed according to published methods [22]. The time course was examined initially at the same time points as mentioned previously in this article. Subsequently, MCs were exposed to two minutes of stretch at −18 kPa with and without agonists and antagonists as mentioned previously in this article. Static MCs exposed to acute PMA and A23187 as described previously in this article were also studied. After washing in cold PBS, nuclear extracts of stretched cells were prepared by lysis in hypotonic buffer (20 mmol/L HEPES, pH 7.9, 1 mmol/L EDTA, 1 mmol/L EGTA, 20 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L $Na_4P_2O_7$, 1 mmol/L DTT, 0.5 mmol/L PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.6% Nonidet P-40) and were homogenized and sedimented at 16,000 *g* for 20 minutes at 4°C. Pelleted nuclei were resuspended in hypotonic buffer with 0.42 mol/L of $NaCl_2$ and 20% glycerol and rotated for 30 minutes at 4°C. After centrifugation for 20 minutes at 16,000 × *g*, the supernatant was collected, and the protein concentration was measured with the Bio-Rad assay kit.

Activator protein-1 consensus oligonucleotides were prepared by incubating 2 μL consensus oligonucleotide (1.75 pmol/μL), 1 μL T4 Polynucleotide Kinase 10 × buffer, 1 μL [γ-32P]ATP (3000 Ci/mL; DuPont, Boston, MA, USA) and 5 μL nuclease-free water for 10 minutes at 37°C. The reaction was stopped by adding 1 μL of 0.5 mol/L EDTA. Unlabeled ^{32}P-ATP was removed from the oligonucleotide mixture with D-25 Sephadex columns.

The supernatants were used as nuclear proteins for the binding assay. Three micrograms of nuclear proteins were incubated with 2 μg of poly(dI-dC).poly(dI.dC) (Pharmacia, Uppsala, Sweden) in binding buffer (20 mmol/L HEPES, pH 7.9, 1.8 mmol/L $MgCl_2$, 2 mmol/L DTT, 0.5 EDTA, 0.5 mg/mL BSA), incubated for 30 minutes at room temperature, and then reacted with radiolabeled consensus oligonucleotides at room temperature for 20 minutes (50,000 cpm to 100,000 cpm). Reaction mixtures were electrophoresed in a 6% polyacrylamide gel and were autoradiographed. Competition experiments were performed with unlabeled AP-1 consensus oligonucleotides in 100× excess.

RNA isolation and semiquantitative reverse transcriptase-polymerase chain reaction for fibronectin

Total RNA from MCs was isolated by the single step method of Chomczynski and Sacchi [23] as we have published [18, 24]. Briefly, after two hours of stretch (−18 kPa) or the addition of agonists (30 minutes) with the static MC protocol, cells were washed and scraped into 4 mol/L guanidinium isothiocyanate; 0.1 mol/L 2-mercaptoethanol was added to the cell suspension and vortexed; 0.2 milliliters of 2 mol/L Na acetate, 2.0 mL of diethyl pyrocarbonate-treated phenol, and 0.4 mL of chloroform were then sequentially added. The solution was iced, centrifuged, and then precipitated with isopropanol at −20°C for 60 minutes. Total RNA was pelleted, resuspended

in 4 mol/L guanidinium isothiocyanate, 0.1 mol/L 2-mercaptoethanol, and isopropanol, and again precipitated at −20°C for 60 minutes. The purity and concentration were determined by measuring the optical densities at 260 and 280 nm prior to use. The A260/280 ratio ranged from 1.75 to 1.95. Subsequently, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as we have reported [24]. One microgram of total RNA was mixed with 10 U RNAsin and 300 pmol random hexamers. The mixture was heated to 65°C for five minutes and cooled on ice. One hundred units of Moloney mouse leukemia virus reverse transcriptase (M-MLV-RT) and 4 μL of 5 × RT buffer was added, and the mix was incubated at 42°C for two hours. The reaction was stopped by heating at 95°C for five minutes. Product was diluted to 100 μL with deionized water and was kept at −70°C.

For amplification, 2.5 μL of RT product were mixed with 7.5 μL of PCR mix containing 0.1 μmol/L of each of the primer pairs [25, 26] and 2 U of *taq* polymerase. The sample was placed onto a Perkin-Elmer DNA Thermal Cycler (Model 480) and heated to 94°C for four minutes followed by 30 temperature cycles (94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute). β-Actin was coamplified to standardize the amount of RNA subjected to reverse transcription at each time point. Linearity of amplification was ensured for each product by subjecting RT product to amplification through 26, 28, 30, 32, and 34 cycles.

Polymerase chain reaction products were separated on a 1% agarose gel containing ethidium bromide and were photographed and quantitated with a GS 300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA, USA) utilizing a MacIntosh Classic II (System 7.0) and Dynamax HPLC Method Manager (version 1.2).

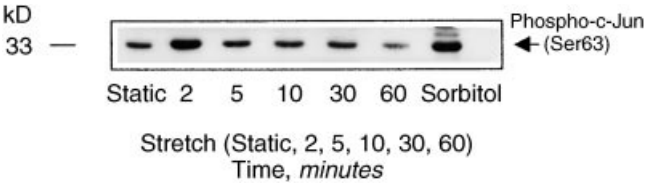

Fig. 1. SAPK/JNK activity time course. Mesangial cells (MCs) were exposed to cyclic mechanical strain (−18 kPa, 60 Hz) for the times indicated. Isolated protein was then incubated overnight with c-Jun fusion protein beads. Recovered pellets were reacted for 30 minutes at 30°C for the kinase assay, electrophoresed, blotted to a nitrocellulose membrane, probed overnight with a phospho-specific c-Jun antibody, and visualized with an HRP-conjugated secondary antibody. Application of mechanical strain led to a prompt increase in SAPK/JNK activity, which was maximal at two minutes. Sorbitol (sorb.) served as a positive control. Four separate experiments were performed. Only the two-minute time point was different from static, and all other groups using analysis of variance with Bonferroni *t*-test ($P < 0.02$).

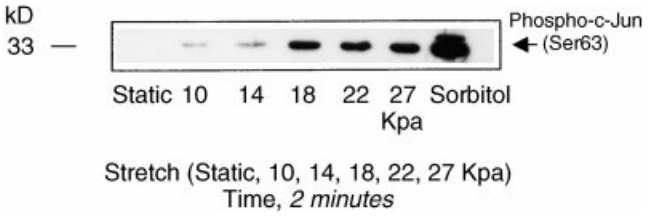

Fig. 2. SAPK/JNK activity stretch magnitude. MCs were exposed to cyclic mechanical strain at the magnitudes of stretch indicated for two minutes at 60 Hz. Isolated protein was then incubated overnight with c-Jun fusion protein beads. Recovered pellets were reacted for 30 minutes at 30°C for the kinase assay, electrophoresed, blotted to a nitrocellulose membrane, probed overnight with a phospho-specific c-Jun antibody, and visualized with an HRP-conjugated secondary antibody. Application of mechanical strain led to an increase in SAPK/JNK activity that was maximal at −27 kPa. Sorbitol (sorb.) served as a positive control. Four separate experiments were performed. All magnitudes of stretch showed significant increases over static by analysis of variance with Bonferroni *t*-test ($P < 0.05$).

RESULTS

Characterization of stretch-induced SAPK/JNK activity in MCs

The first aim of the current study was to determine the time and magnitude of stretch dependence of SAPK/JNK activation in MCs. MC lysates were initially subjected to Western blot analysis of SAPK/JNK expression, and subsequently, analysis of SAPK/JNK activity was performed by determination of the phosphorylation of a c-Jun fusion protein by MC lysates. Cultures were exposed to strain for periods of 2, 5, 10, 30, and 60 minutes at vacuum levels of −10, −14, −18, −22, and −27 kPa. Unstretched MCs were used as controls. Representative autoradiographs are shown in Figures 1 and 2. In Figure 1, the time course was explored as MCs were stretched at −18 kPa for the times indicated previously. No changes in the protein expression of either the 46 or 54 kD isoform of SAPK/JNK was observed (data not shown). However, Figure 1 shows that changes were seen in SAPK/JNK activity at two minutes, which returned promptly to static values by five minutes. In Figure 2, dependence on the magnitude of stretch was studied, as MCs were exposed to the levels of vacuum outlined previously in this article for two minutes. No changes in the protein expression of either the 46 or 54 kD isoform of SAPK/JNK was observed (data not shown). In contrast, Figure 2 shows a representative autoradiograph of the magnitude of stretch dependence of SAPK/JNK activity. Increases in SAPK/JNK activity were seen at all levels of stretch, but sharply increased activity was reached at −18 kPa and above. A negative pressure of −18 kPa corresponds to an average 16% increase in the surface diameter. While there are no data concerning the acute increases in glomerular surface diameter in models of glomerular capillary hypertension (such as the 5/6 nephrectomy model), the rise in transcapillary pressure gradient is in the order of 20%, and it would

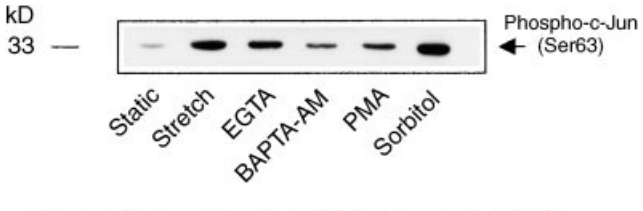

Fig. 3. Mediators of stretch-induced SAPK/JNK activity. MCs were exposed to cyclic mechanical strain of −18 kPa for two minutes at 60 Hz. Cells were preincubated with egtazic acid (EGTA) 1 mmol/L or BAPTA-AM (10 μmol/L) for 30 minutes prior to the application of stretch to chelate extra and intracellular calcium, respectively, and with phorbol 12-myristate 13-acetate (PMA) 100 nmol/L for 24 hours prior to stretch to inactivate PKC. Sorbitol (sorb) was used as a positive control. Isolated protein was then incubated overnight with c-Jun fusion protein beads. Recovered pellets were reacted for 30 minutes at 30°C for the kinase assay, electrophoresed, blotted to a nitrocellulose membrane, probed overnight with a phospho-specific c-Jun antibody, and visualized with an horseradish peroxidase (HRP)-conjugated secondary antibody. BAPTA-AM and PMA both down-regulated SAPK/JNK activity, whereas EGTA was without significant effect. Four separate experiments were performed. Only inhibition with BAPTA-AM and PMA was different from control and EGTA by analysis of variance with Bonferroni *t*-test (<0.05).

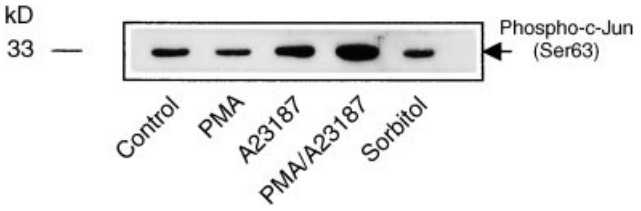

Fig. 4. Agonist-induced SAPK/JNK activity in static cells. Unstretched MCs were studied to determine the effects of increased intracellular calcium and protein kinase C (PKC) activity on the SAPK/JNK activity. Cells were incubated with the calcium ionophore A23187 or 1 μmol/L PMA for 30 minutes (to increase PKC activity). Isolated protein was then incubated overnight with c-Jun fusion protein beads. Recovered pellets were reacted for 30 minutes at 30°C for the kinase assay, electrophoresed, blotted to a nitrocellulose membrane, probed overnight with a phospho-specific c-Jun antibody, and visualized with an HRP-conjugated secondary antibody. A23187 led to an increase in SAPK/JNK activity at 30 minutes, which was markedly potentiated by activation of PKC. Four separate experiments were performed. Stimulation with A23187 or A23187 + PMA was significantly different from control (static) and PMA alone by analysis of variance with Bonferroni *t*-test ($P < 0.02$).

seem unlikely the surface diameter would increase by more than this. Consequently, −18 kPa was chosen for further study. Accordingly, stretch protocols of two minutes and −18 kPa were used for subsequent experiments.

Characterization of mediators of SAPK/JNK response

Subsequently, we sought to determine the role of calcium and PKC in the prompt induction of SAPK/JNK seen when MCs were exposed to cyclic stretch. To ensure that alterations in SAPK/JNK protein level could not be contributing to any observed activity changes, we initially performed Western blot analysis of SAPK/JNK protein in MCs stretched for two minutes at −18 kPa preincubated with EGTA (1 mmol/L) or BAPTA-AM (10 μmol/L) for 30 minutes prior to the application of stretch to chelate extra- and intracellular calcium, respectively, and with PMA 100 nmol/L for 24 hours prior to stretch to inactivate PKC. Neither the protein expression of the 46 kD nor the 54 kD isoform of SAPK/JNK was affected by these antagonists (data not shown). In sharp contrast to this, Figure 3 shows SAPK/JNK activity as measured by phosphorylation of a c-Jun fusion protein in response to stretch with the addition of antagonists of calcium and PKC as mentioned previously in this article. Preincubation with EGTA (1 mmol/L) to chelate extracellular calcium did not affect the SAPK/JNK response to MC stretch. However, the activation of SAPK/JNK was abrogated by chelation of intracellular calcium with BAPTA-AM (10 μmol/L) and diminished substantially by down-regulation of PKC by 24-hour preincubation with 100 nmol/L PMA.

To confirm these observations, we then explored the effects of increased intracellular calcium and PKC activity on the SAPK/JNK response in unstretched MCs. Neither preincubation with the calcium ionophore A23187 or 1 μmol/L PMA for 30 minutes to increase PKC affected SAPK/JNK protein expression (data not shown). When SAPK/JNK activity was studied under the same conditions (Fig. 4), the addition of A23187 increased the SAPK/JNK activity in MCs, and this was further potentiated when PMA and A23187 were added together, suggesting a synergistic effect of intracellular calcium and PKC activity. Control experiments were performed in order to ensure that SAPK activation by acute PMA and A23187 was similar on fixed, type 1 collagen-coated, plastic-bottomed plates and flexible but unstretched, type 1 collagen-coated, rubber-bottomed plates. Figure 5 shows that SAPK activity in unstretched MCs in response to PMA or A23187 was similar in both types of plates.

Nuclear protein binding to AP-1 consensus sequences

We next sought to determine whether downstream intranuclear events were affected by stretch and manipulation of calcium and PKC by assessing the binding of nuclear protein to AP-1 consensus sequences. Binding in response to stretch application to MC at −18 kPa for 2, 5, 10, and 30 minutes was studied (Fig. 6). Concordant with our observations of SAPK/JNK activity in response to stretch, maximally increased nuclear protein binding was seen after two minutes of stretch ($N = 4$ experiments). An increase in binding was observed through 10 minutes, however. Excess cold AP-1 consensus sequences abrogated the binding seen in response to stretch, confirming the specificity of the assay. To determine whether antagonists affected nuclear protein binding to AP-1 consen-

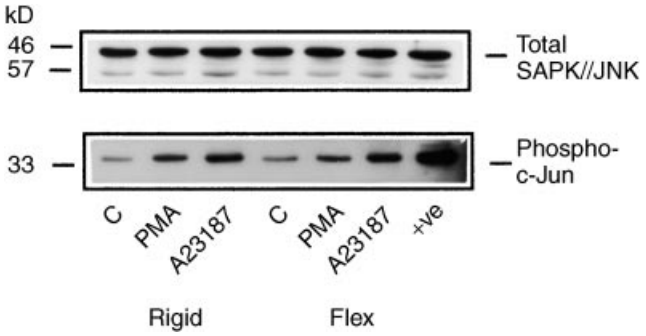

Fig. 5. Effect of plate composition. Unstretched MCs were studied to determine the effects of increased intracellular calcium and PKC activity on the SAPK/JNK activity and to ensure that the composition of the collagen-coated well base did not affect activity. Cells were incubated with the calcium ionophore A23187 or 1 μmol/L PMA for 30 minutes (to increase PKC activity). Isolated protein was then incubated overnight with c-Jun fusion protein beads. Recovered pellets were reacted for 30 minutes at 30°C for the kinase assay, electrophoresed, blotted to a nitrocellulose membrane, probed overnight with a phospho-specific c-Jun antibody, and visualized with an HRP-conjugated secondary antibody. Identical results were seen on either flexible or rigid-bottomed plates ($N = 3$ experiments). The positive (+) lane is sorbitol 400 mmol/L for 30 minutes as an osmotic stimulus.

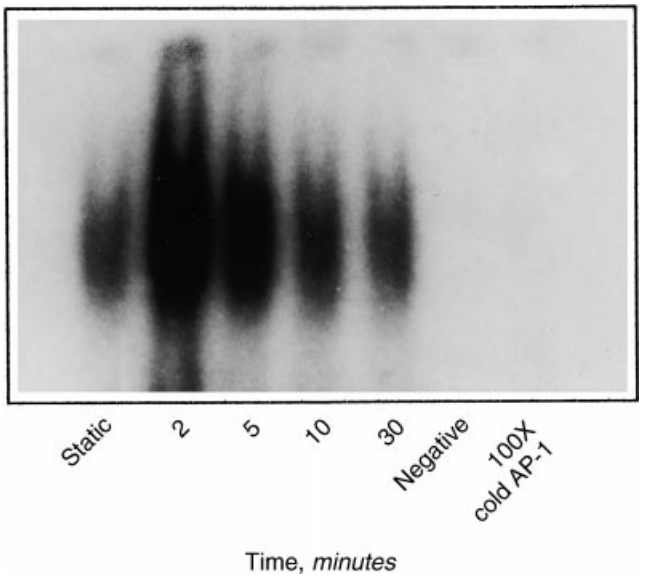

Fig. 6. Nuclear protein binding to activated protein-1 (AP-1) consensus sequences in response to stretch. MCs were exposed to mechanical strain (−18 kPa, 60 Hz) for the times indicated. AP-1 gel shift assay was performed with isolated nuclear protein. Nuclear proteins were incubated with poly(dI-dC).poly(dI.dC) and then reacted with radiolabeled consensus AP-1 oligonucleotides, electrophoresed, and autoradiographed. Retardation of the label, indicating binding of nuclear protein, was seen in stretched cells, maximally at two minutes. No retardation of radiolabel was seen in the absence of nuclear protein (neg). Specificity was ensured with competition experiments using excess unlabelled AP-1 consensus oligonucleotide, which revealed no retardation of the label (extreme right lane). Four separate experiments were performed, and a representative autoradiograph is shown.

sus sequences, MCs were exposed to EGTA, BAPTA-AM, and PMA, as mentioned previously in this article, prior to the application of stretch. The results are shown in Figure 7. Both PMA and BAPTA-AM decreased binding, whereas EGTA was without effect, which is concordant with our observations of SAPK/JNK activity under the same conditions. Again, excess cold AP-1 consensus oligonucleotides were successful in competing off nuclear protein. In static cells, AP-1 nuclear protein binding also mirrored SAPK activity changes. Both acute PMA and A23187 increased nuclear protein binding to AP-1 (data not shown). To confirm further the specificities of gel-shift assays, experiments were performed with excess cold consensus nuclear factor-κB (NF-κB) oligonucleotides. No retardation of the label was seen in these experiments (data not shown).

RNA isolation and semiquantitative RT-PCR for fibronectin

To relate early signaling events to ECM protein expression, fibronectin mRNA levels were determined in static MCs, stretched MCs, and static MCs exposed to acute PMA or the calcium ionophore A23187. Figure 8 shows that fibronectin mRNA levels appear to be increased by stretch, acute PMA, and A23187, within the limits of this qualitative assay ($N = 4$).

DISCUSSION

Mechanical stresses trigger intracellular events in several cell lines, including ECM gene expression and proliferation [27]. In the kidney glomerulus, MC responses to pathologic levels of physical force (which may be as little as a 20% increase in capillary pressure) may result in sclerosis [8, 9, 12]. In vitro studies of stretched MCs have demonstrated that this stimulus results in the production of collagenous proteins [2] and fibronectin [13] when compared with static cells, and that this is associated with a rise in TGF-β signaling activity [12, 13, 28]. MC proliferation has also been observed in response to stretch [2, 29, 30].

The first site of transduction of mechanical signals occurs at the cell membrane. Events here have been studied in several cell lines exposed to mechanical strain. In MCs, stretch-induced TGF-β expression is tyrosine kinase dependent, suggesting that tyrosine kinase receptor ligands such as platelet-derived growth factor (PDGF) may play a role in mechanotransduction of ECM protein [13]. The proliferative effects of mechanical force are to some extent matrix dependent, with cells adherent to fibronectin showing the greatest response, and this is inhibited by blocking integrins with arginine-glycine-aspartate (RGD) peptides [31]. Cyclic strain might also lead to cytoskeletal rearrangement mediated by integrin-focal adhesion complex interactions, and tyrosine phosphorylation of the focal adhesion-associated kinase $pp125^{FAK}$

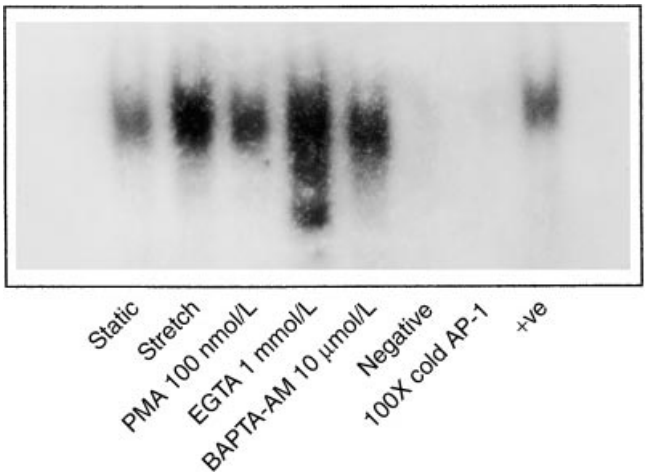

Fig. 7. Mediators of nuclear protein binding to AP-1 consensus sequences in response to stretch. MCs were exposed to cyclic mechanical strain of −18 kPa for two minutes at 60 Hz. Cells were preincubated with EGTA (1 mmol/L) or BAPTA-AM (10 μmol/L) for 30 minutes prior to the application of stretch to chelate extra and intracellular calcium, respectively, and with PMA 100 nmol/L for 24 hours prior to stretch to inactivate PKC. AP-1 gel shift assay was performed with isolated nuclear protein. Nuclear proteins were incubated with poly(dI-dC).poly(dI.dC) and then reacted with radiolabeled consensus AP-1 oligonucleotides, electrophoresed, and autoradiographed. Retardation of the label was seen in response to stretch. EGTA did not affect this, but less retardation was observed in the presence of BAPTA-AM or with the down-regulation of PKC by PMA. No retardation of radiolabel was seen in the absence of nuclear protein (neg). Specificity was ensured with competition experiments using excess unlabelled AP-1 consensus oligonucleotide, with no retardation of label (right lane) and use of an unrelated NF-κB consensus oligonucleotide, which did not compete with the label (+ve lane). Four separate experiments were performed, and a representative autoradiograph is shown.

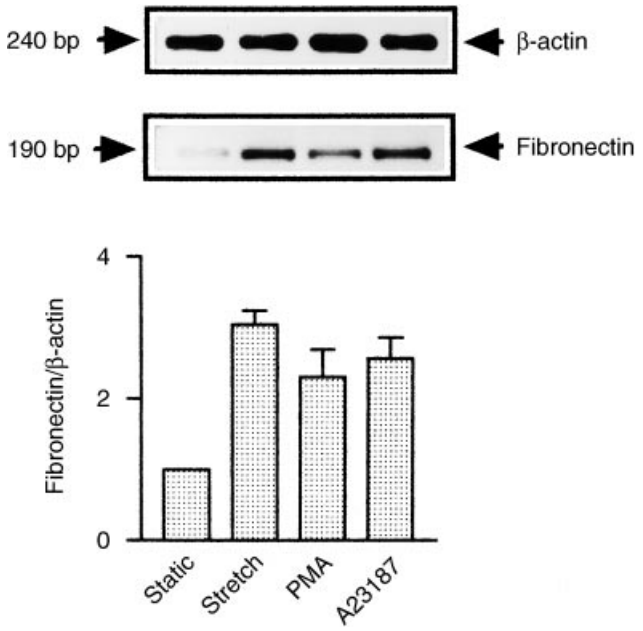

Fig. 8. (*A*) Fibronectin message levels in response to stretch or agonists. Fibronectin mRNA levels were measured by RT-PCR after stretch (2 h) or in static cells exposed to PMA 100 nmol/L to activate PKC or to the calcium ionophore A23187 for 30 minutes. The housekeeping gene β-actin was amplified to control for RNA isolation, RT, and PCR. Stretch and agonists increased both fibronectin message levels significantly when compared with unstimulated cells. Four separate experiments were performed. (*B*) The data from four separate experiments are shown graphically. The mean and standard deviation error bars were derived from four separate experiments.

is seen in stretched MCs [32]. Ion channels also play a role at the cell membrane. In VSMCs, stretch activated phospholipase C in a calcium-dependent manner [33].

Signaling of mechanical stimuli to the cell nucleus after membrane events involves the ubiquitous MAP kinase cascades. Each of the MAP kinase cascades consists of three protein kinases acting sequentially a MAP kinase kinase activator (MEKK), a MAP kinase activator (MEK), and a MAP kinase [34]. We have previously reported that high levels of mechanical strain (−28 kPa) activates p42/p44 MAPK and p38 MAPK in primary cultured glomerular MCs at 30 minutes [18], and in the current study, we demonstrate that mechanical strain also activates the SAPK/JNK pathway in a dose- and time-dependent manner at a time point earlier than we observed for p42/p44 MAPK and p38 MAPK in this previous work [18]. Furthermore, the magnitude of strain required for activation was less than seen for p42/44 and p38 MAPKs [18]. Maximal activity was observed after two minutes of cyclic stretch/relaxation, with −18 kPa vacuum, corresponding to a maximum increase of 22% in the diameter of the cells in culture. SAPK/JNK has been demonstrated to respond rapidly to mechanical strain in VSMCs [35], cardiac fibroblasts [27], cardiac myocytes [20], and aortic endothelial cells [36]. In MCs, JNK-SAPK is not activated in response to a constant (that is, nonpulsatile) pressure load [17].

SAPK/JNK was first described in 1990 [37] and was found to be strongly activated in most cell types by inflammatory cytokines, especially tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [38], and by the physical stress of heat shock [38]. The pattern of SAPK/JNK activity seen in our studies mirrors the response of this kinase cascade to Ang II seen in neonatal cardiac myocytes, in which Ang II stimulates SAPK/JNK in a calcium and PKC-dependent manner [21]. However, paracrine secretion of Ang II would seem very unlikely to account for the observed changes, given the rapidity with which it occurs after application of stretch. Furthermore, the Ang II-induced SAPK/JNK activation is not PKC dependent [39], and we found that activation of SAPK/JNK by mechanical strain was suppressed by either the down-regulation of PKC or the chelation of intracellular calcium. An increase in intracellular calcium (A23187) was sufficient to activate SAPK/JNK in our static MCs, but the simultaneous addition of PMA for 30 minutes to activate PKC and A23187 led to a marked increase in SAPK/JNK activation. Chelation of intracel-

lular calcium was recently found to prevent c-*fos* induction in response to growth factors such as PDGF in cultured MCs, suggesting a dependence of the kinase signaling pathways on calcium, which is consistent with our observations [40]. In T lymphocytes, SAPK/JNK is also activated by these two major signaling pathways [41] so that second messengers involved in SAPK/JNK activation may be dependent on the cell type.

The upstream mediators of SAPK/JNK activation were not investigated in the current study, but MEKK1 may be involved, by analogy to the effect of Ang II in cardiac myocytes [21]. Interestingly, PKC-β has been implicated in a pathway involving PKC-MEKKI-SEK1-SAPK in a myeloid leukemia cell line [42]. In addition small GTP-binding proteins of the Rho family, Rac1 and Cdc42, have been shown to regulate the activation of SAPK/JNK [43]. This pathway (Cdc42-MEKKI-SAPK) has been implicated in the activation of SAPK/JNK by PMA and gonadotropin-releasing hormone in a pituitary cell line [44]. Further studies are necessary to relate stretch-mediated SAPK activation to Rho-related GTPases in MCs.

Downstream, the JNK-SAPK pathway phosphorylates and activates c-Jun [38]. Phosphorylated c-Jun may then complex with c-Fos to form the AP-1 transcription factor or complex with ATF-2. The ATF-2/c-Jun dimer binds to promoters containing cAMP-responsive element consensus sequences and may therefore activate a different array of genes than AP-1 [45]. Accordingly, we related the activation of SAPK/JNK to increases in nuclear proteins binding consensus AP-1 sequences. Coincident with the kinase activation, there was an increase in protein binding to AP-1 consensus oligonucleotides in MCs, and down-regulation of PKC and chelation of intracellular calcium could partially abrogate this increase. In static cells, nuclear protein binding to AP-1 consensus sequences increased after acute PMA and A23187, implicating calcium and PKC as possible mediators in the strain-induced activation of SAPK. Since several workers have shown that cyclical stretch leads to MC production of collagenous proteins [2] and fibronectin [13] when compared with cells grown in static cultures and that TGF-β expression is increased [12, 13, 28], it is tempting to speculate that transactivation of ECM protein genes, growth factor genes, and the stimulation of MC proliferation may be dependent, at least in part, on AP-1 [2, 29, 30]. Increased AP-1 binding to oligonucleotides was more sustained than the brief SAPK activation that we observed, suggesting that brief "pulses" of kinase activation may be sufficient to result in sustained transcriptional effects. Our observation that fibronectin mRNA levels increased in stretched MCs supports this hypothesis.

In conclusion, our study establishes that mechanical strain delivered as cyclic stretch induces prompt SAPK/JNK activation in MCs. This effect is dependent on intracellular calcium and PKC. SAPK/JNK activation is accompanied by an increase in AP-1 binding, linking an environmental stimulus to the regulation of gene expression.

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APPENDIX

Abbreviations are: AP-1, activator protein-1; Ang II, angiotensin II; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ECM extracellular matrix; EGTA, egtazic acid; FCS, fetal calf serum; HRP, horseradish peroxidase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MCs, mesangial cells; MEK, MAP kinase activator; MEKK, MAP kinase kinase activator; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcriptase-polymerase chain reaction; SAPK/JNK, jun N-terminal kinase/stress-activated protein kinase; SDS-PAGE, sodium-dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TGF-β1, transforming growth factor-β1; VSMCs, vascular smooth muscle cells.

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